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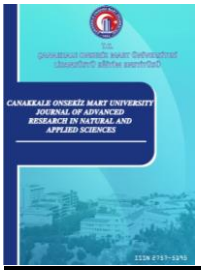

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Evaluation of Proteins Released to Medium in Yeast-Bacteria Co-culture System

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Research Article

Abstract – Cultivation of microorganisms in ideal laboratory conditions seperates them from their natural conditions and isolates them from their microbial world, especially from their competitors. With traditional pure culture-oriented cultuvation techniques, interactions mediated by small molecules are not taken into account, resulting in the precise nature of the interactions being largely unknown. Co-culture systems are systems in which two or more different cell populations are grown together. In this way, studies on natural interactions between populations can be made and synthetic interactions that are not observed in nature can be provided. With these systems, natural product discovery, microbial ecology, evolution and pathogenesis studies are carried out. In addition, co-culture systems are also used in industrial, environmental and medical studies. In this study, the wild strain of *Schizosaccharomyces pombe* and the DH5α strain of *Escherichia coli* were grown in their own specific media, then cultured for 48 hours and 72 hours by cultivating in media containing 0,1% glucose with different cell number, and finally the differentiation in the proteins released by the cells into the medium was observed in SDS polyacrylamide gels. Different from the control conditions, new protein bands that emerged under the co-culture conditions were detected and two of these bands were analyzed by mass spectrometry (MS). While 6 of differentaited proteins were released by *S.pombe*, 257 proteins matched with *E.coli* proteom. These proteins are; Various carbohydrate-binding proteins, membrane proteins involved in the identification of various signaling molecules and antibiotics, and other proteins involved in various cellular processes.

Keywords *– Co-culture, Escherichia coli, proteomics, Shizosaccharomyces pombe*

1. Introduction

Microbial interactions that take place in the natural environment are critical to the survival of organisms. These natural interactions are mediated by a variety of molecules such as secondary metabolites, signal-sensing molecules, and peptides (Fuqua et al., 2001; Waters et al., 2005). Possible interactions between two microbial populations can be considered positive interactions (commensalism, protocooperation, and mutualism), negative interactions (amensalism and competition), and interactions that are positive for one and negative for the other (parasitism and predation) (Bailey and Ollis, 1986; Atlas and Bartha, 1998). An example of these relationships is that in passive competition, one species restricts the access of other species to nutrients by secreting digestive enzymes or siderophores (iron chelators, produced to provide access to insoluble iron) (Diggle et al., 2007; Rendueles and Ghigo, 2012; Griffin et al. 2004; Scholz and Greenberg, 2015). Active competition, on the other hand, is a form of competition in which rival cells actively or through chemical warfare harm each other to eliminate each other. This form of competition occurs through the production of antimicrobials ranging from bacteriocins to peptides and antibiotics (Chao and Levin, 1981; Riley and Gordon, 1999; Ghoul and Mitri, 2016). Antimicrobial compounds can mediate competition between different species,

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between related species of the same species, as well as between genetically identical individuals in a population.

Co-culture systems are systems that contain mixed cultures that allow two or more different cell populations to be grown together to study intercellular interactions. The main reasons for conducting experiments with these systems are; The study of natural interactions between populations can be listed as increasing the success of culture for some populations and establishing synthetic interactions between populations. Co-culture systems are used to study the interactions between cell populations, for natural product discovery, to increase product productivity obtained by biotechnological studies, and to improve culture behaviours; It has been used for a long time in basic scientific studies, industrial and medical studies (Goers et al., 2014). In addition, studies such as discovering infection mechanisms (Cottet et al., 2002), attempts to make artificial tissue, and finding and developing drug-specific compounds (Morae et al., 2012; Kim, 2005) is effectively carried out with co-culture systems. With future industrial, medical, or environmental co-culture applications in mind, many synthetic biology systems have been developed. Some of these can be summarized as bacterial cells designed to kill pathogens, some therapeutic applications (Bermùdez-Humaràn et al., 2011), and tumor targeting (Anderson et al., 2008).

By applying and modifying the co-culturing technique, new peptides with previously undetected antimicrobial properties can be isolated. In this way, new drug-active ingredients can be found and developed. Moreover, with the co-culturation technique, previously undetected interactions between organisms can be detected and new unstudied pathways unique to the organism can be discovered.

In this study, it was tried to determine the differentiation in the proteins released into the medium under co-ulture conditions of the wild strain of *Schizosaccharomyces pombe*, a eukaryotic single-celled model organism frequently used in the field of molecular biology, and *Escherichia coli* (DH5α), an example of a prokaryotic organism. The new protein bands, which appeared different from the control conditions of the co-culture, were determined by SDS polyacrylamide gel electrophoresis, and then the proteins belonging to these bands were determined by the Mass Spectrometry method. The proteins released into the medium were mostly found to belong to E. coli and these proteins were classified and analysed based on Gene Ontology (GO).

2. Materials and Methods

2.1. Organisms, Media, and Establishment of Co-culture System

In this study, wild type (972 h-) of *Schizosaccharomyces pombe* Lindner liquifaciens, a unicellular and eukaryotic yeast species belonging to the Ascomycetes class of the Fungi kingdom and *Escherichia coli* DH5α strains, were used. *S. pombe* wild strain (972 h-) and *E. coli* DH5α strain used in the study were obtained from the culture collection of the Faculty of Science, Department of Molecular Biology and Genetics in Istanbul University. While Luria-Bertani (LB, pH 7.2) medium is used for the production and storage of E. coli, Yeast Extract Solid Medium (YEA) is used for the production and storage of *S. pombe*, and Yeast Extract Liquid (YEL) medium, containing 3% Glucose is used for propagation. Co-cultur, on the other hand, was established in YEL medium and this medium contained 0.1% glucose to provide increased competition.For co-culture establishment, *S. pombe* cells stored in solid YEA at +4 °C were first seeded into a new YEA by streaking technique and allowed to grow at 30 °C for 2 days. Cells grown in YEA were seeded in 5 mL YEL with an initial cell number of 106 cells/ml and allowed to grow overnight in a shaking oven at 30 °C, 180 rpm, and co-culture cultivation was performed.

E. coli cells stored in liquid LB at +4°C were seeded into fresh 5 ml LB and allowed to grow at 37°C. For E. coli cells not to pass into the stationary phase, the OD should not exceed 0.6 at 600 nm. At 200 µl OD 600 nm, the number of *E. coli* cells with a value of 0.5 corresponds to approximately 106 h/ml. OD measurements of *E. coli* cells, which were inoculated in LB and allowed to grow at 37 °C, were made at certain hourly intervals using sterile LB as a blank, and co-culture cultivation was started when the OD value was approximately 0.5.

Pure cultures of *S. pombe* and *E. coli* were used separately as a control group in co-culture experimental sets. Co-culture was started with 2.106 h/ml S. pombe and 106 h/ml *E. coli*. For the *S. pombe* control group, 2.106 h/ml S. *pombe* cells were inoculated in 10 ml of YEL medium containing 0.1% glucose, for the *E. coli* control

group, 106 h/ml *E. coli* was inoculated into 10 ml of YEL medium containing 0.1% glucose. For the co-culture set, 2.106 h/ml *S. pombe* and 106 h/ml *E. coli* cells were seeded in 10 ml of YEL medium containing 0.1% glucose. All sets were incubated in an orbital shaker at 32 °C, 180 rpm.

E. coli cells stored in liquid LB at +4°C were seeded into fresh 5 ml LB and allowed to grow at 37°C. For *E. coli* cells not to pass into the stationary phase, the OD should not exceed 0.6 at 600 nm. The OD of 200 µl culture is 0.5 at 600 nm, and the number of E. coli cells corresponds to approximately 106 h/ml. OD measurements of *E. coli* cells grown in LB at 37 °C were made at certain hourly intervals and co-culture cultivation was started when the OD value was approximately 0.5.

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2.2. Extraction and Analysis of Proteins

At the end of 48 and 72 hours, cultures were transferred to sterile falcons. The falcon tubes were centrifuged at 5000 rpm for 5 minutes and the supernatant was taken into a sterile falcon tube and removed from the precipitated cells.

Protein precipitation was performed according to the method suggested by Wessel and Flügge (1984) from the supernatant (extracellular space) that was purified from cells after centrifugation. This method was the methanol-chloroform-water method, which is a method applied for the quantitative recovery of protein in a dilute solution in the presence of detergent and lipids. To obtain protein, the supernatant was divided into sterile falcons with a 1 ml sample in each falcon. 4 ml of methanol was added to 1 ml of the sample and the tube was vortexed. Then, 1 ml of chloroform was added to the tube and mixed again with the help of a vortex. Then 3 ml of dH_2O was added and after vortexing again, it was centrifuged at 10000g for 10 minutes. After centrifugation, the supernatant up to the 2^{nd} phase was removed from the samples, which were separated into 3 phases. (At this stage, the proteins are collected in the second phase.) Then, 4 ml of methanol was added to the tube and mixed again by vortexing. It was centrifuged again at 10000g for 10 minutes. The supernatant was completely removed, and the methanol was allowed to evaporate. Subsequently, the precipitated protein samples were dissolved in 50-200 µl of 1% SDS. The SMART™ BCA (bicinchoninic acid) Protein Assay Kit (iNtRON Biotechnology) was used to determine the resulting protein concentrations and followed the manufacturer's instructions. The absorbance of the samples obtained from the last step was measured in triplicate for all samples and standards using a spectrometer device (EON, BioTek Instruments Inc.) at a wavelength of 562 nm. A standard curve was created using the concentration and absorbance values of BSA standards. Protein concentrations were calculated by inserting the absorbance values of the protein samples into the equation obtained from the standard curve.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%) was used to separate the proteins by denaturing the water-soluble proteins suggested by Walker (2002). Mini-gel electrophoresis system (Mini-PROTEAN® Tetra Cell, Bio-Rad) was used for SDS-PAGE application. Electrophoresis was performed at constant voltage (200 V) and separation was continued until the band formed by bromophenol blue reached the lower edge of the gel. After gel electrophoresis, the Coomassie brilliant blue staining method was used to make the protein bands visible. After SDS-PAGE was completed, the gel was placed in a clean, acid-proof plastic container.

The staining solution was poured into the gel container until it completely covered the gel, and the container was shaken at 50 rpm on an orbital platform shaker (Heidolph Unimax 1010) for two hours at room temperature. The staining solution used at this stage was 0.05% (w/v) Coomassie brilliant blue R-250 (Merck 112553), 50:10:40 (v:v:v) MeOH (Methanol, Sigma-Aldrich 24229) : AcOH (Acetic Acid, Sigma-Aldrich 27225)) : It was prepared with a mixture of dH_2O.

After two hours, the gel was taken into the wash solution [prepared with a mixture of MeOH: AcOH: dH2O at the ratios of 5: 7: 88 (v: v: v)] and shaken at 50 rpm until free of dye. The wash solution was changed periodically at this stage, and the washing process was repeated until the non-banded portions of the gel were completely free of dye. The bands formed in the gel were visualized on the ChemiDoc MP (Bio-Rad) imaging system using ImageLab 5.2.1 software.

After imaging, the protein bands of the co-culture, which differed from the control groups, were cut with the help of a sterile scalpel and taken into dH2O, and their analysis were carried out by LC/MS-MS, Acıbadem LabMed company affiliated to Acıbadem University. The results with protein ID numbers obtained from LC/MS-MS were analysed using UniProt and PANTHER programs. While the names and functions of the proteins were searched on the UniProt website, metabolic functions and the cellular components of the proteins were analysed using the PANTHER program.

3. Results and Discussion

3.1 Electrophoretic Analysis Results

Three samples from the 48th hour of the co-culture (15 µg protein), and the three samples from the 72nd hour (90.4 µg protein) were loaded into the wells. Proteins run at a constant voltage of 200 V were then visualized by the Coomassie staining method. Visible bands on the gel were analyzed using the ChemiDoc MP (Bio-Rad) system and ImageLab 5.2.1 software (Figure 1). Since the bands 1 and 2 marked in Figure 1 were found to differ qualitatively from the 72nd hour control groups, these bands were cut with a sterile loop and transferred to a clean microcentrifuge tube in dH_2O for LC-MS/MS analysis.

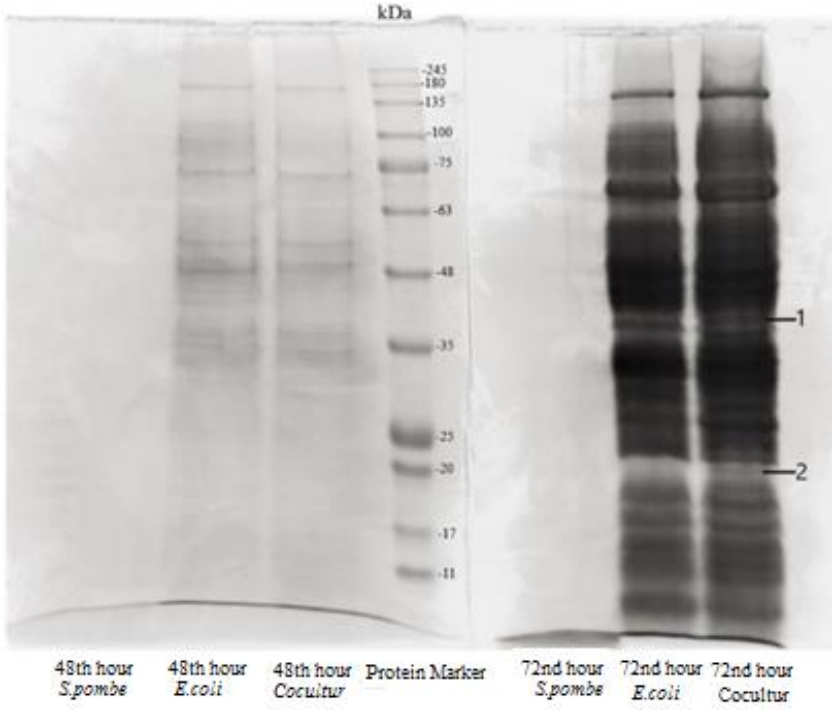

Figure 1. The protein bands of the 48th hour samples were weaker than the bands of the 72nd-hour samples.

It was determined that the bands 1 and 2 marked in the figure differed qualitatively from the 72nd hour control groups. For this reason, these bands were cut with a sterile loop and placed in a clean microcentrifuge tube in dH_2O and prepared for LC-MS/MS analysis.

3.2 Identification of proteins

The proteins identified as a result of LC-MS/MS analysis were evaluated in UniProt and the names and functions of the proteins are given as an appendix. Here, 6 protein-coding genes in the bands of the proteins released into the medium by the co-culture were found in the S.pombe genome and were classified according to their biological functions and cellular locations using the online platform PANTHER (Figure 2). Other 257 protein-coding genes were found in the *E. coli* genome. These proteins were also classified according to their cellular location (Figure 3) and their cellular functions (Figure 4).

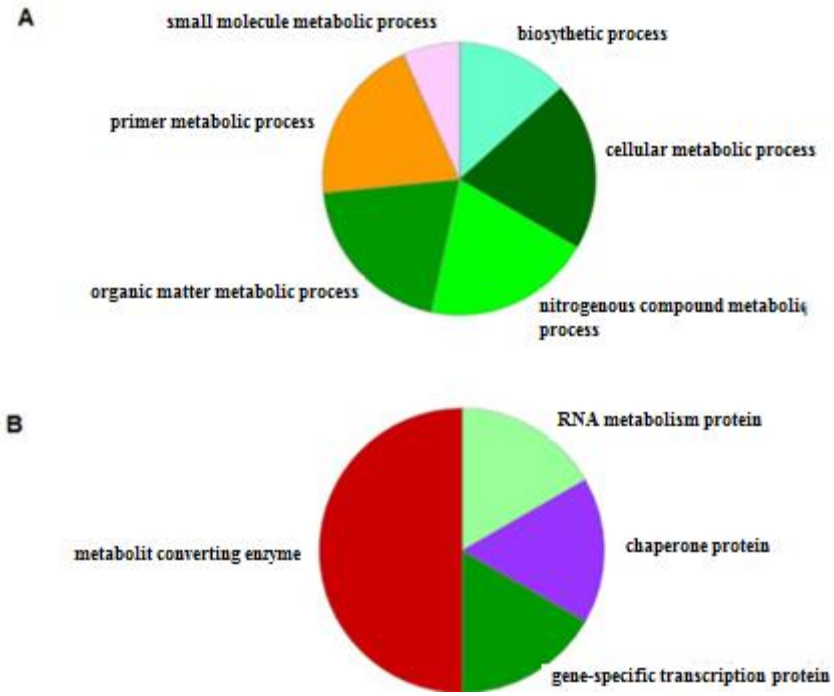

Figure 2. Classification of proteins identified in the *S.pombe* genome according to their biological functions (A) and cellular location (B).

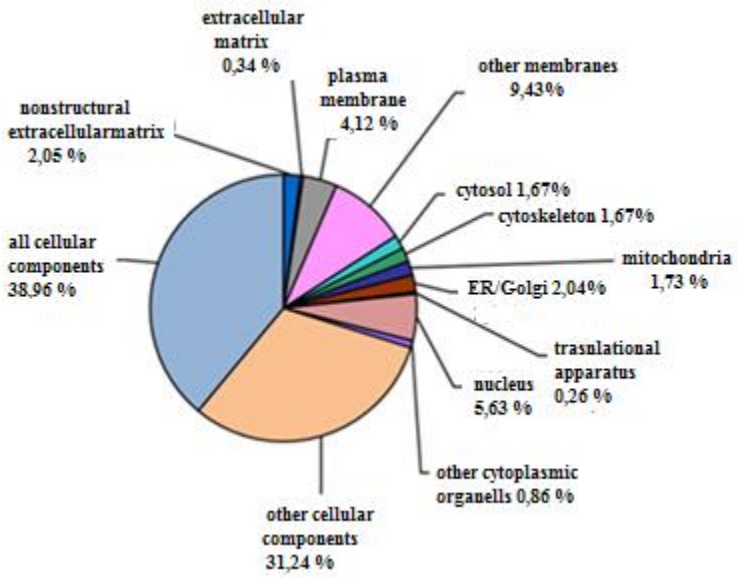

Figure 3. Classification of proteins identified in the *E. coli* genome according to their cellular location

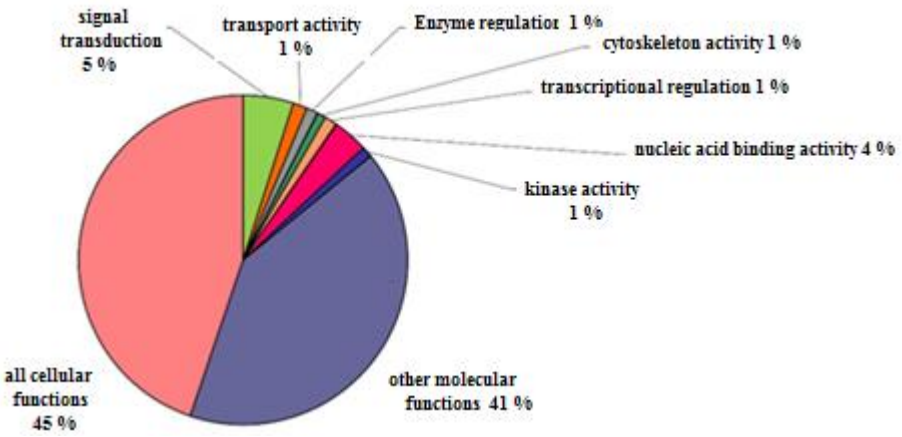

Figure 4. Classification of proteins identified in *E. coli* according to their biological functions.

Maltose/maltodextrin-binding periplasmic protein (malE) and D-galactose-binding periplasmic protein (mglB) found in *E. coli* cells are among the binding proteins found in LC-MS-MS results. The binding protein MglB has a high affinity for glucose (Ferenci, 1996), and at low glucose concentration, glucose is mainly taken up by cells in this way. In a study by Egli et al. (1993), giving 1.8 mg l−1 galactose to a low glucose-containing *E. coli* culture decreased the glucose utilization of the cells and led to glucose accumulation in continuous culture. In addition, Hua and., (2004) found that the expression of a series of genes encoding membrane transporter proteins was significantly induced in cultures of limited chemostats for various carbon sources, and that about half of these genes were maltose (all genes of malGFE and malK-lamB-malM operons), galactose. They showed that there are genes involved in a wide variety of high-affinity ABC transport systems that facilitate uptake (three genes of the mglBAC operon). In *E. coli*, the ABC transporter gene family covers approximately 5% of the entire genome (Linton and Higgins, 1998). This shows how important these genes are. Carbon-limited cultures have been proposed to investigate bacterial growth and behavior under environmental conditions (Matin, 1979; Moriarty, 1993; Morita, 1993). Therefore, as a result of the proteome analysis performed in our study, the qualitative increase of mglB and malE proteins in cultures containing 0.1% glucose and limited carbon source suggests that *E. coli* compete for the source under co-culture conditions.

The DNA protection protein, dps, is formed as a result of the RpoS-dependent state during nutrient starvation, during the transition to the stationary phase, and forms a complex with DNA, giving it resistance to being affected by DNase (Almiron et al., 1992). The co-culture of *E. coli* bacteria used in the study before they entered the stationary phase and the analysis of the proteins obtained from the cells obtained at 72 hours, as a result of the analysis of the dps formation, gives results consistent with the current calorie restriction. In addition, dps protein is also involved in the response to oxidative stress, iron and copper toxicity, heat stress and acid-base shocks (Nair and Finkel, 2004). The glycerol dehydrogenase enzyme makes it possible for microorganisms to use glycerol as a carbon source under anaerobic conditions (Gonzalez et al., 2008; Silva et al., 2019). SodA is an important enzyme in the response to oxidative stress (McCord, 1993), and this Sod enzyme functions as a metalloprotein with Fe and/or Mn (Edwards et al., 1998). Quinone reductase can reduce toxic chromate to the less toxic Cr^{+3}, while generating very low amounts of ROS (Ackerley et al., 2004). Only Gonzalez et al., (2005) stated in their study that quinone reductase can be tolerant to ROS by two electron transfer. The presence of dps, sodA[Mn], gldA proteins in the co-culture medium indicates that oxidative stress occurs in addition to starvation stress in *E. coli* and that the response mechanisms are activated.

Another protein obtained in the study; YggE is an uncharacterized protein. The yggE gene is thought to be one of the genes that show high expression under oxidative stress, and yggE gene products are thought to be functional in protecting bacterial cells or ameliorating cellular damage caused by oxidative stress (Levina et al., 1999). According to the results of the study by Kim et al. (2005); The yggE gene product is thought to protect genes involved in amino acid biosynthesis from oxidative damage by reducing intracellular ROS to a moderately harmless level. Considering that the co-culture conditions in this study exposed the cells to stress in many

respects (glucose starvation, presence of another type of organism, heat stress caused by the production of cells at 32° C, etc.), the increase in the production of yggE protein supports previous studies.

In our study, it was observed that porin and its associated outer membrane proteins, Omp proteins, were also released into the medium. On the extracellular side there are broad, extending loops between the filaments, while on the periplasmic side there are short loops. Thanks to these features, Omps provides high stability in the membrane and the ability to fight against harsh environments (Rollauer et al., 2015). Although different Omps have different sequences and functions, they are similar in terms of structural and biological features (Chaturvedi and Mahalakshmi, 2017). OmpA was first identified as a heat exchangeable protein in *E. coli* in 1974 (Foulds and Chai, 1978) and was first purified in 1977. The molecular mass ranges from 28 kDa to 36 kDa (Chai and Fould, 1977). The OmpA family is a group of porin proteins that have a high copy number in the Omps of gram-negative bacteria. OmpA plays an important role in maintaining bacterial surface integrity (Samsudin and., 2016). OmpX belongs to a family of highly conserved proteins that appear to be important for virulence by neutralizing host defense mechanisms (Heffernan et al., 1994). OmpA, OmpX, OmpC, OmpF, FocD, FimC proteins are the other proteins that appear in LC-MS-MS results in 72 hours of co-culture. Dekoninck et al., (2020) showed that the OmpA protein is effective in the Rcs stress response. Laubacher and Ades (2021) stated that the Rcs response also exhibits the function of creating resistance against beta-lactam antibiotics. In addition, Dam et al., (2017) showed that the MicC sRNA molecule controls OmpC/N and thus takes part in the response to beta-lactam antibiotics. Moreover, OmpC functions as a receptor in the recognition of signalling molecules in contact-dependent interaction (Beck et al., 2016). The present findings show that *E. coli* and *S. pombe* are in contact in the culture medium, due to the presence of cysteine synthase A protein and the stimulation of this protein in contact-dependent growth inhibition (Diner et al., 2012). Dupont et al., (2007) demonstrated that OmpX and OmpF are functional in the early response to environmental stress. Choi and Lee (2019) stated that OmpC is involved in both the antibiotic response and the integrity of the cell membrane. Ghai et al., (2018) reported that OmpF can show selective permeability to molecules of similar size depending on their structure and dipolar properties, and that the chemical stability of ampicillin, a widely used antibiotic, is an important factor in penetrating OmpF to reach its target in the periplasm. The formation and functionalization of the fimbria, which allows the bacteria to settle on the outer surface, depends on FocC and FocD (Klemm et al., 1994,1995). FimC also acts as a chaperone in fimbria biogenesis (Choudhury et al., 1999).

Bacteria are organisms commonly used in biotechnological processes. They are widely used in medicine, food and environmental industries, especially due to the various enzymes they secrete out of the cell. The enzyme N-ethylmaleimide reductase breaks down toxic pollutants such as 2,4,6-trinitrotoluene (TNT). NemA reduces nitro groups on the aromatic ring with NfsA and NfsB. Thus, *E. coli* uses nitrogen for cell growth (González-Pérez and., 2007). Hexavalent Chromium (Cr VI) is a serious environmental pollutant and is highly soluble in water in this form. Bacteria have been discovered that convert Cr VI to its less toxic and insoluble form, Cr III. Robins et al. (2013) discovered in a study that E. coli NemA is a Cr VI reductase that works with high efficiency.

N-ethylmaleimide reductase (NemA), one of the proteins determined to be released into the medium in our study, is one of the important proteins encountered in biotechnology (González-Pérez et al., 2007). Different bioprocesses are encountered in the production of such related proteins and their feasibility is being done. Co-culture systems are highly adaptable to biotechnological studies. If advanced molecular analyzes and feasibility of products that are thought to have increased expression with co-culture systems are discovered, and it is discovered that more efficient products with lower costs are obtained, it can contribute to biotechnological studies by producing enzymes such as NemA with high efficiency. In the light of the findings, we can argue that the formation of the above-mentioned proteins by *E. coli* in the co-culture medium is a response to the stresses caused by environmental conditions and *S. pombe*.

The most important limitation of many microbial competition studies conducted to date is that data from in vitro studies could not be tested in more natural environments. Studies with pure cultures do not consider the interaction of secondary metabolites and provide a limited perspective for understanding the behaviour of microorganisms against each other (Park et al., 2011; Nai and Meyer 2018). In this context, the use of co-cultures in in vitro studies and the creation of natural environment conditions are very important for the

progress of these studies. The establishment and development of co-culture systems will contribute to answering various biological questions and overcoming various biological problems.

Properties such as the resilience of microorganisms that cannot be easily constructed in a single microorganism, the stability of metabolism in changing environments and the changes in their responses to stress, their tolerance to toxic metabolic wastes or compounds in the raw material can be understood with co-cultures (Zhu et al., 2012).

4. Conclusion

This study includes the establishment of *S. pombe* and *E. coli* co-cultures and the examination of the proteins that are released into the culture medium. As the main finding, it can be said that competition among organisms increases the amount and types of protein released. Considering that antibiotic resistance developed by microorganisms poses a serious threat today, obtaining peptide-antibiotics that can be produced by co-culturation gains importance. According to our results, although mechanisms that are responsible for the production of antimicrobial peptides seemed activated, we failed to detect these kinds of peptides in this condition. To increase the competition in the co-culture system and to encourage cells to produce antimicrobial peptides, options such as keeping the cultures in media with a lower energy source for a longer time and using media with different compositions can be tried. Accordingly Different alternative co-culture options can be tested by changing the cell numbers of both species. After SDS-Page, the study can be expanded by analyzing other bands that are qualitatively different. The results obtained in this study may be important as preliminary findings that can contribute to basic science with the changes in the metabolism of *S. pombe* and *E. coli* microorganisms in the co-culture system, and to future biotechnological studies with the products they produce as a result of these changes.

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Author Contributions

Ayşegül Yanık: Methodology, Investigation.

Çağatay Tarhan: Methodology, Investigation, Analysis, Writing - Original Draft, Writing - Review

& Editing, Supervision

Conflicts of Interest

The authors declare no conflict of interest.

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